

Lol p I-induced IL-4 and IFN- γ production by peripheral blood mononuclear cells of atopic and nonatopic subjects during and out of the pollen season

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*The reciprocal effects of IL-4 and IFN- γ on IgE synthesis have been well established. It has also been shown that these two lymphokines are secreted by different subsets of CD4⁺ T cells (T_{H1} and T_{H2}), and that T_{H2} helper T lymphocytes could be involved in the pathophysiology of allergic diseases. But little is known about the effects of an allergen on the profile of lymphokine synthesis by human peripheral blood mononuclear cells (PBMCs) of allergic and nonallergic subjects. We studied the production of IL-4 and IFN- γ by PBMCs of atopic and nonatopic donors after in vitro stimulation by the group I allergen from *Lolium perenne* pollen (Lol p I), during and out of the grass pollen season. On natural exposure to pollen, Lol p I-induced IL-4 production was observed only with atopic donors (6 of 8), whereas the synthesis of IFN- γ was observed for all nonatopic donors (7 of 7) and most allergic patients (5 of 7). At the time of the study, higher amounts of IFN- γ were produced by PBMCs of nonatopic donors than by PBMCs of atopic patients. Out of the pollen season the production of IL-4 was not observed either by atopic (n = 11) or by nonatopic subjects (n = 5). On the other hand, IFN- γ was produced by PBMCs of most subjects (atopic, 10 of 11; nonatopic, 5 of 5), but at the time of the study no difference was observed between the two groups. These results show that Lol p I induces different profiles of IL-4 and IFN- γ production by PBMCs of atopic and nonatopic subjects. In atopic subjects this profile of lymphokine synthesis is influenced by the natural exposure to pollen, which is in keeping with the seasonal rise of IgE antibodies. (J ALLERGY CLIN IMMUNOL 1993;91:950-6.)*

Key words: Lol p I, IL-4, IFN- γ , atopic

Atopic patients are distinguished from nonatopic subjects by a high production of IgE antibodies. Recently the effects of interleukin (IL)-4 and interferon (IFN)- γ on IgE synthesis have been demonstrated. Indeed, in mice it has been shown that IL-4 induces IgE and IgG1 production by liposaccharide-stimulated B-cell blasts,^{1,2} and this effect is inhibited by in vivo administration of an antibody directed to IL-4,^{3,4} sup-

Abbreviations used

ELISA:	Enzyme-linked immunoabsorbent assay
IL:	Interleukin
IFN- γ :	Interferon-gamma
Lol p I:	Group I allergen from <i>Lolium perenne</i> pollen
MAb:	Monoclonal antibody
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
SI:	Stimulation index

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porting the specificity of this finding. On the other hand, IFN- γ was shown to inhibit this IL-4-induced IgE synthesis.^{5,6} In human beings it has been reported that IL-4 could induce peripheral blood mononuclear cells (PBMCs) of atopic and nonatopic donors to produce IgE and IgG4 isotypes,⁷⁻¹⁴ whereas this effect is inhibited by IFN- γ .^{8,9,15,16}

In the last few years, on the basis of the profile of lymphokines secreted, subsets of CD4⁺ T cells have been distinguished. In mice, T_{H1} cells express mRNA for and secrete IL-2, IFN- γ , and IL-1 β but not IL-4, IL-5, IL-6, and IL-10, whereas T_{H2} cells secrete IL-4, IL-5, IL-6, and IL-10 but not IL-2, IFN- γ , and IL-1 β .¹⁷⁻²⁰ Other lymphokines such as IL-3 and granulocyte-macrophage colony-stimulating factor are secreted by both sets. Recently it has been shown that similar subsets could also exist in human beings.²¹⁻²³ Because T_{H2} cells produce IL-4, it has been postulated that these cells are present in atopic subjects and are involved in the elevated IgE production.

It has been reported that PBMCs of atopic patients produced aberrant levels of IL-4 and IFN- γ on in vitro stimulation with polyclonal activators.²⁴ They synthesized higher levels of IL-4 and lower levels of IFN- γ than PBMCs of healthy donors tested in parallel. However, the effects of an allergen on IL-4 and IFN- γ synthesis by PBMCs of atopic and nonatopic subjects have never been analyzed. The aim of the present study was to determine the patterns of IL-4 and IFN- γ synthesis by PBMCs of atopic and nonatopic donors after in vitro stimulation by the group I allergen from *Lolium perenne* (*Lol p I*). We report that for the atopic subjects the natural exposure to the allergen influence the in vitro production of IL-4 and IFN- γ .

METHODS

Cell donors

Fifteen adult atopic donors were selected from the Allergy and Immunology clinic of Centre Hospitalier de l'Université Laval (CHUL) on the basis of a history of seasonal allergic rhinitis, positive skin prick test (Omega Laboratories, Montréal, Canada), positive radioallergosorbent test (RAST; Pharmacia, Piscataway, N.J.) to *Lolium perenne*, and no previous immunotherapy. Four atopic donors were studied during the pollen season alone, four during and out of the pollen season, and seven subjects out of the pollen season alone. Twelve nonatopic donors (seven studied during the pollen season and five studied out of the pollen season) without any symptoms of seasonal allergic rhinitis, negative skin prick test, and RAST to common inhalants were also included in this study.

Allergen preparation

Lol p I was purified from crude extracts (Omega Laboratories) by a reverse immunosorbent column as described previously.²⁵ In brief, crude extracts were dialyzed against phosphate-buffered saline (PBS), concentrated and passed through a Sepharose CL-4B column (Pharmacia) coupled to purified monoclonal antibody (MAb) (290A-167) directed to *Lol p I*; the MAb was prepared in our laboratory as reported in other studies.²⁵⁻²⁷ The column was washed out, absorbed material was eluted with 3 mol/L MgCl₂ and dialyzed against PBS and finally concentrated to 1 mg/ml

with use of an Amicon (Amicon, Division of W.R. Grace Co., Beverly, Mass.) YM5 ultrafilter (cut-off of molecular weight 5000). The purity of the eluted material was assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

IL-4 and IFN- γ determinations

The quantitative determinations of IL-4 and IFN- γ were performed with use of commercial enzyme-linked immunosorbent assay (ELISA) kits in accordance with the instructions of the manufacturer. Intertest-4 from Genzyme (Boston, Mass.) was used to measure IL-4, whereas IFN- γ was measured with IFN- γ ELISA kit from Endogen (Boston, Mass.). Sensitivity of the assays were 45 pg/ml for IL-4 and 5 pg/ml for IFN- γ . When no cytokines were detectable in the supernatants, the value of sensitivity of the assay was attributed for these specific experiments.

Culture method

PBMCs were isolated from heparinized venous blood of the subjects by density gradient centrifugation as previously described.²⁸ The cells were washed three times in Hank's balanced salt solution (Gibco, Grand Island, N.Y.) and resuspended in complete medium composed of RPMI 1640 supplemented with glutamine 1%, penicillin 100 U/ml, streptomycin 100 μ g/ml, and 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah). Unfractionated PBMCs (1.5×10^5 cells/well) were cultured (37° C, 5% CO₂) in triplicate wells (200 μ l/well) of 96-well round-bottomed plates (Nunc, Kamstrup, Denmark) in complete medium alone or in presence of *Lol p I* (10 μ g/ml). Ten micrograms per milliliter of *Lol p I* was determined to be the optimal concentration in preliminary studies (unpublished observation). After 48 hours of incubation, the cells were harvested, and supernatants were collected and stored at -70° C until used.

Assay for in vitro proliferation

Unfractionated PBMCs (1.5×10^5 cells/well) were cultured (37° C, 5% CO₂) in triplicate wells (200 μ l/well) of 96-well round-bottomed plates in absence or in presence of different concentrations of *Lol p I*. Six days later, 1 μ Ci/well (methyl [³H]) TdR (DuPont, New England Nuclear, Boston, Mass.) was added. After 16 to 18 hours cells were harvested, and radioactivity was measured with use of a liquid scintillation counter (Beckman, model LS5000; Beckman Instruments, Inc., Fullerton, Calif.). Stimulation indexes (SI) were calculated as follows:

$$SI = \frac{\text{CPM in presence of } Lol p I}{\text{CPM in absence of } Lol p I}$$

Statistical analysis

Results are expressed in means of cytokine (picograms per milliliter) $\pm$ SEM and compared with the unpaired Student's *t* test. The comparison of the response obtained with use of the same subjects during and out of the pollen season was performed with the paired Student's *t* test.

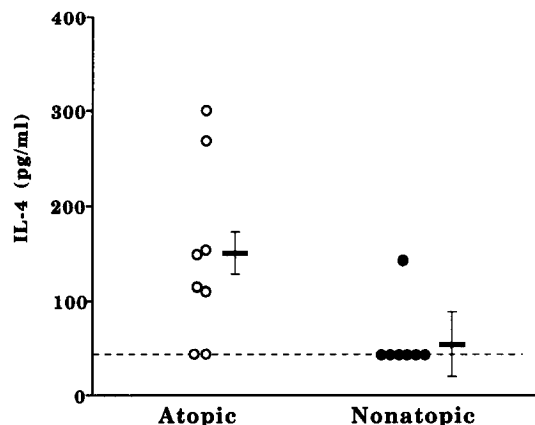


FIG. 1. *Lol p I*-induced IL-4 synthesis by unfractionated PBMCs from atopic ($n = 8$) and nonatopic donors ($n = 7$) during the pollen season. For each different subject, data are expressed in means of triplicate cultures; (---) limit of sensitivity of assay; (■) reveals mean $\pm$ SEM values from both groups.

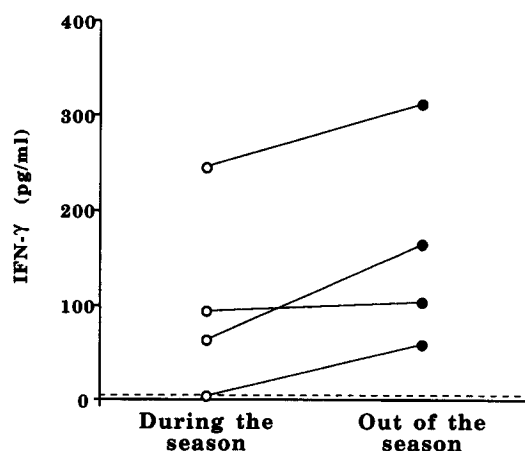


FIG. 2. *Lol p I*-induced IFN- γ synthesis by unfractionated PBMCs from atopic ($n = 7$) and nonatopic donors ($n = 7$) during the pollen season. For each different subject, data are expressed in means of triplicate cultures; (---) limit of sensitivity of assay; (■) reveals mean $\pm$ SEM values from both groups. Mean IFN- γ concentration was significantly higher ($p \leq 0.008$) for the nonatopic group compared with atopic group.

RESULTS

Lol p I-induced IL-4 production: atopic versus nonatopic donors

The production of IL-4 by PBMCs of atopic and nonatopic donors after in vitro stimulation by *Lol p I* was studied during and out of the pollen season. Spontaneous IL-4 production was not observed either during or out of the pollen season for any of the different subjects included in this study. However, in the presence of *Lol p I*, detectable levels of IL-4 could be

measured in the different supernatants. The *Lol p I*-induced IL-4 production during the pollen season is plotted in Fig. 1. As can be seen, a significant number of allergic subjects (6 of 8) produced IL-4 in presence of *Lol p I*, whereas only 1 of 7 nonatopic donors did produce detectable amounts. Mean concentrations of IL-4 were 147.7 ± 22.93 pg/ml and 58.76 ± 35.17 pg/ml, respectively. Notice that the value of sensitivity of the assay (45 pg/ml) was attributed when no cytokine was detected in the supernatants. Out of the pollen season assays were performed on 11 atopic and five nonatopic donors for the production of IL-4 induced by *Lol p I*. At the time of the study none of these subjects produced IL-4 when PBMCs were pulsed by the allergen. We noticed that for atopic donors the production of IL-4 in presence of *Lol p I* was significantly higher ($p \leq 0.0001$) during the pollen season than out of the season, whereas for nonatopic donors the *Lol p I* did not induce IL-4 production either during or out of the pollen season.

Lol p I-induced IFN- γ production; atopic versus nonatopic donors

As has been observed for IL-4, no spontaneous IFN- γ production was measured either for the atopic or for the nonatopic subjects at any time in the study. During the pollen season IFN- γ production was observed for all nonatopic donors (7 of 7) and most allergic subjects (5 of 7), as shown in Fig. 2. The mean concentrations were 84.68 ± 22.20 pg/ml for the atopic group and 312.05 ± 86.04 pg/ml for the nonatopic group. In these conditions it is interesting to notice that a significantly higher level of IFN- γ ($p \leq 0.008$) was produced by PBMCs of nonatopic donors than by PBMCs of atopic subjects. Fig. 3 shows that out of the pollen season the production of IFN- γ was also measured in most supernatants tested. Ten of 11 atopic donors produced IFN- γ with a mean concentration of 240.54 ± 54.23 pg/ml, whereas 5 of 5 supernatants of nonatopic donors contained detectable levels of IFN- γ with a mean concentration of 274.60 ± 62.15 pg/ml. In contrast with the results obtained during the pollen season, at the time of the study no significant difference in the IFN- γ production ($p \geq 0.2$) was observed. It is interesting to notice that the production of IFN- γ by PBMCs of atopic donors was significantly enhanced ($p \leq 0.01$) out of the season (Fig. 3) when compared with the production observed during the pollen season (Fig. 2), whereas for the nonatopic donors no significant difference ($p \geq 0.2$) was observed between the two periods of assay.

Proliferation response of PBMCs to *Lol p I*

Lol p I-induced proliferation assays were performed out of the pollen season with use of PBMCs of atopic

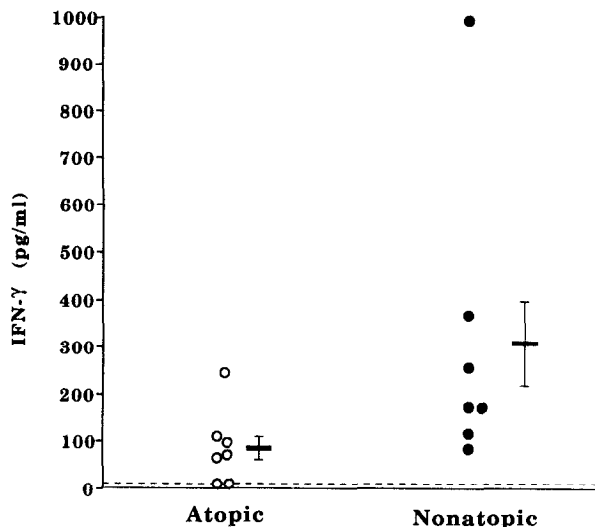


FIG. 3. *Lol p* I-induced IFN- γ synthesis by unfractionated PBMCs from atopic ($n = 11$) and nonatopic donors ($n = 5$). Out of the pollen season. For each different subject, data are expressed in means of triplicate cultures; (---) limit of sensitivity of assay; (■) reveals mean $\pm$ SEM values from both groups. No significant difference ($p \geq 0.2$) was observed between the mean IFN- γ concentrations measured for the atopic group and for the nonatopic group.

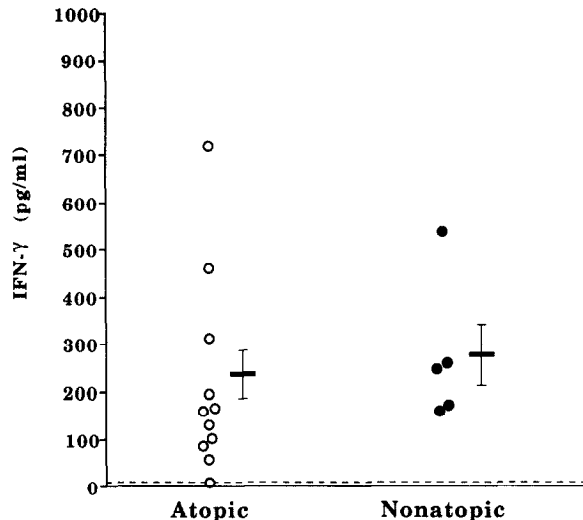


FIG. 4. Proliferative responses to *Lol p* I (100 ng/ml) of PBMCs from different atopic and nonatopic donors. Data are expressed as SI, described in Methods.

and nonatopic subjects. Fig. 4 shows the results for the nonatopic subjects ($n = 5$) and for the atopic patients ($n = 5$). It can be seen that both groups responded to *Lol p* I. No significant difference was observed between the mean of the SI from the two groups (not shown). No correlation was seen between the SI of PBMCs and the levels of cytokines.

***Lol p* I-induced IL-4 and IFN- γ production: atopic patients during versus out of the pollen season**

To confirm the observation that for the atopic group an inverse pattern of cytokine production was observed out of the pollen season, it was necessary to study the response obtained with use of the same PBMC donors at each period of assay. Then PBMCs of four atopic patients were used to study the *Lol p* I-induced cytokine production both during and out of the pollen season. Although a significant IL-4 production was observed during the season, it is interesting to notice that out of the season this response was not stimulated by *Lol p* I, because none of the supernatants contained detectable amounts of IL-4. Fig. 5 shows that IFN- γ production by PBMCs of these four subjects was significantly higher ($p \leq 0.005$) out of the pollen season than during the grass pollen season. These results support the observation that the natural exposure to pollens could in-

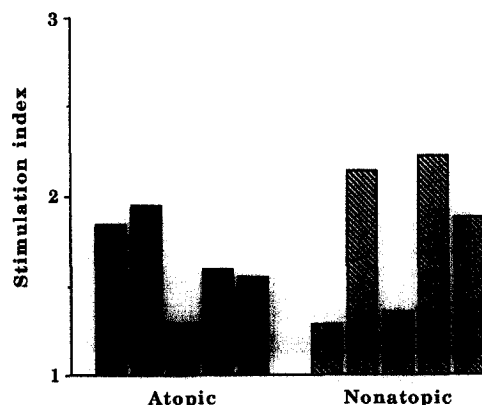


FIG. 5. *Lol p* I-induced IFN- γ synthesis by PBMCs of atopic subjects studied during and out of the pollen season ($n = 4$); (---) limit of sensitivity of assay. Significantly higher IFN- γ production ($p \leq 0.005$) was observed when the cultures were performed out of the pollen season.

fluence the allergen-induced lymphokine production by PBMCs of atopic donors.

DISCUSSION

If IgE antibodies could play a role in the defense against offending agents gaining access through the mucocutaneous linings, they are best known as mediators of allergic diseases. Indeed, elevated levels of these antibodies are found in atopic patients.²⁹⁻³² Recently the role of IL-4 and IFN- γ as the main regulatory cytokines of IgE production with opposite effects has been established. It is interesting to note that over the last few years, in mice and human beings, it has been established that these two lymphokines are produced by different subsets of helper T lympho-

cytes, T_{H1} producing IFN- γ and T_{H2} producing IL-4. From these observations it was tempting to speculate that increased IgE synthesis in atopic subjects could be explained by higher proportion of T_{H2} subset producing IL-4 and promoting IgE synthesis. In fact, it has been shown that the percentage of randomly cloned phytohemagglutinin-activated $CD4^+$ T-cell clones that produce IL-4 without substantial IFN- γ is higher in atopic than in nonatopic subjects.³³ Recently different groups have reported that allergen-specific T-cell clones established from PBMCs of atopic donors bear most of the T_{H2} phenotype.²¹⁻²³ In contrast, with use of PBMCs from the same donors, most T-cell clones specific to bacterial components such as tetanus toxoid or protein purified derivative of *Mycobacterium tuberculosis* carried the T_{H1} phenotype.²² It is interesting to note that Wierenga et al.^{21,23} showed that in nonatopic donors house dust mite *Dermatophagoides pteronyssinus* (Dp)-specific T-cell clones were T_{H1} , whereas they were T_{H2} in atopic donors. In subjects allergic to timothy grass pollens, skin biopsies obtained from the sites of *Phleum pratense*-induced late-phase cutaneous reaction showed an infiltration of cells expressing mRNA for IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor.³⁴ This observation supports the possibility of an involvement of T_{H2} lymphocytes in the pathophysiology of allergic diseases. In addition, it has recently been reported that T_{H2} lymphocytes are predominant in bronchoalveolar lavage of subjects with atopic asthma as compared with control subjects.³⁵

Up to now, most studies on IL-4 and IFN- γ producing T cells have been performed with use of T-cell clones derived from atopic and nonatopic donors as discussed above. Few studies have been performed with PBMCs, which is more representative of the in vivo situation than T-cell clones. Rousset et al.²⁴ have studied the mitogenic stimulation of unfractionated PBMCs of atopic and nonatopic donors. They observed that PBMCs of atopic patients produced higher levels of IL-4 and lower levels of IFN- γ than PBMCs of healthy donors after in vitro stimulation with polyclonal activators. These results are not in conflict with those observed from T-cell clones. Herein we report for the first time the production of IL-4 and IFN- γ by PBMCs of atopic and nonatopic subjects after in vitro stimulation by a purified allergen, the *Lol p I*. With use of this system, our results, obtained during the season, confirm that PBMCs of atopic subjects are inducible to produce IL-4 on activation by an allergen, whereas PBMCs of nonatopic donors are not. On the other hand, it is also interesting to notice that during the pollen season IFN- γ was produced by PBMCs of both atopic and nonatopic subjects. How-

ever, lower levels were produced by atopic patients than by nonatopic donors. These results confirm that atopic and nonatopic donors produce different cytokines on stimulation by an allergen, as suggested by the observations of Rousset et al.²⁴

It has been shown that in atopic subjects the serum levels of specific IgE enhanced during the pollen season reach a maximum after the season and decrease thereafter.³⁶ These observations suggest that this phenomenon could be related to a higher production of allergen-induced IL-4 in atopic patients during the pollen season. To study this point, the experiments were also performed out of the season. It is interesting to note that although during the season PBMCs of atopic patients were producers of IL-4 on allergenic stimulation, out of the season no detectable amount of this cytokine was measured. Moreover, the production of IFN- γ by cells of atopic subjects was significantly higher out of than during the pollen season. Then out of the season no significant difference was seen for IFN- γ between atopic and nonatopic donors. These results strongly suggest that natural exposure to pollens influence the balance between T_{H2} and T_{H1} specific lymphocytes in peripheral blood of atopic subjects by enhancing the proportion of T_{H2} cells leading to increased production of IL-4 and consequently IgE. Since Robinson et al.³⁵ have shown that the T_{H2} T lymphocytes are present in bronchoalveolar lavage of atopic subjects, it is tempting to speculate that during the pollination period such cells are stimulated at the respiratory tract level and are further released into the systemic circulation. This possible mechanism furthers our understanding of the clinical observation of the priming effect.

It has been reported that IL-4 inhibits the expression of IFN- γ mRNA and as a consequence inhibits the synthesis of this cytokine.^{37,38} During the season the lower levels of IFN- γ along with significant amounts of IL-4 in atopic subjects could suggest an inhibition of the synthesis of IFN- γ by IL-4 as shown in these systems. This point is presently under investigation. Also, T_{H2} cells have been found to specifically produce a cytokine inhibiting the synthesis of several cytokines by T_{H1} lymphocytes. This cytokine was first called cytokine synthesis inhibitory factor and was further renamed IL-10.³⁹⁻⁴¹ It could therefore be postulated that during the season T_{H2} T lymphocytes, on specific stimulation, produced IL-10, which in turn could down-regulate the IFN- γ synthesis.

The effects of the natural exposure to pollens on the secretion of lymphokines by PBMCs of atopic and nonatopic donors after allergen-specific stimulation have been studied for the first time. Our results show that the profile of lymphokine production by PBMCs

of atopic donors after stimulation by an allergen is different during the pollen season than out of the season, and suggest that the balance between T_H1 and T_H2 cells is influenced by the natural exposure to pollens. During the season our results support the hypothesis that a higher proportion of circulating T_H2 helper T cells could be effectively found in atopic patients and are in keeping with the seasonal rise of IgE antibodies.

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Fibrin formation during ongoing cutaneous allergic reactions: Comparison of responses to antigen and codeine

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Background: Fibrin formation, assessed by fibrinopeptide A levels, was evaluated over a 5-hour period at skin chamber sites challenged continuously with pollen antigen or codeine in 10 reactive individuals.

Methods: The levels of fibrinopeptide A at antigen sites were compared with those at sites challenged with buffer diluent alone or with codeine for the first 3 hours, followed by antigen challenge during the subsequent 2 hours.

Results: Findings showed: (1) fibrinopeptide A levels were higher at antigen challenge sites than at codeine challenge sites by the third hour, with these levels at both sites greater than those at buffer sites; (2) antigen challenge of the previous codeine sites during the third to fifth hours led to a further increase in fibrinopeptide A levels; (3) fibrinopeptide A levels correlated with chamber fluid immunoglobulin G levels but not with chamber fluid histamine levels.

Conclusions: Because antigen and codeine both activate mast cells prominently, these findings suggest that other factors play a role in the persistent fibrin formation at allergic skin reaction sites. Because antigen activates both basophils and mast cells and codeine only activates mast cells, we conclude that both basophils and mast cells contribute to the persistent fibrin formation at sites of allergic reactions. (*J ALLERGY CLIN IMMUNOL* 1993;91:956-60.)

Key words: Coagulation, fibrin, fibrinopeptide A, antigen, codeine, skin chamber, late phase responses, mast cells, basophils, histamine, IgG

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